Mice lacking inducible nitric oxide synthase are not resistant to lipopolysaccharide-induced death

(septic shock/endotoxin/gene targeting/homologous recombination)

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Contributed by Oliver Smithies, August 15, 1995

ABSTRACT   Nitric oxide produced by cytokine-inducible nitric oxide synthase (iNOS) is thought to be important in the pathogenesis of septic shock. To further our understanding of the role of iNOS in normal biology and in a variety of inflammatory disorders, including septic shock, we have used gene targeting to generate a mouse strain that lacks iNOS. Mice lacking iNOS were indistinguishable from wild-type mice in appearance and histology. Upon treatment with lipopolysaccharide and interferon γ, peritoneal macrophages from the mutant mice did not produce nitric oxide measured as nitrite in the culture medium. In addition, lysates of these cells did not contain iNOS protein by immunoblot analysis or iNOS enzyme activity. In a Northern analysis of total RNA, no iNOS transcript of the correct size was detected. No increases in serum nitrite plus nitrate levels were observed in homozygous mutant mice treated with a lethal dose of lipopolysaccharide, but the mutant mice exhibited no significant survival advantage over wild-type mice. These results show that lack of iNOS activity does not prevent mortality in this murine model for septic shock.

In biological systems, nitric oxide (NO) is produced via the oxidation of L-arginine by enzymes known as nitric oxide synthases (NOSs). Three NOS isozymes have been described (for recent reviews see refs. 1 and 2). These include constitutively expressed neuronal (3, 4) and endothelial (5, 6) enzymes and a cytokine-induced enzyme found in macrophages (7), hepatocytes (8), and a variety of other cells (9, 10). Although the biological functions of these enzymes are not completely understood, neuronal NOS is thought to play a role in neurotransmission (11), endothelial NOS is involved in regulation of vascular tone (12, 13), and inducible NOS (iNOS) is involved in immune defense (14, 15). The constitutively synthesized neuronal and endothelial enzymes produce small amounts of NO in response to increases in intracellular calcium levels. In contrast, iNOS is synthesized *de novo* in response to a variety of inflammatory stimuli and produces large amounts of NO over prolonged periods of time (16). NO produced by iNOS has been shown to be beneficial through its antitumor (17, 18) and antimicrobial (15) activities, but it is also thought to cause tissue damage and contribute to pathology in a variety of inflammatory conditions including rheumatoid arthritis (19, 20), inflammatory bowel disease (21), and septic shock (22, 23).

Septic shock is usually the result of a systemic Gram-negative bacterial infection and is characterized by hypotension and the failure of a number of organ systems, especially the liver, kidney, and heart (24). The bacterial membrane component, lipopolysaccharide (LPS), induces the production of host inflammatory mediators such as tumor necrosis factor α, interferon γ (IFN-γ), and interleukin 1β, which in turn cause

an increase in the expression of iNOS. The large amount of NO produced by iNOS has been hypothesized to contribute to LPS-induced hypotension and mortality.

To better understand the biological function of NO produced by iNOS and to determine whether overproduction of NO plays a crucial role in septic shock, we have used homologous recombination in embryonic stem (ES) cells to disrupt the iNOS gene. From these cells we generated mice that lack iNOS. The mice lacking iNOS were then examined in an LPS-induced model of septic shock.

MATERIALS AND METHODS

Design of Targeting Construct. A library of λ bacteriophages carrying fragments derived from a partial *Mbo* I digest of mouse strain 129 genomic DNA was screened with a fragment of the murine iNOS cDNA (25) that contained the calmodulin-binding domain. A λ bacteriophage containing a 14-kb DNA fragment was isolated, from which a 12-kb *Eco*RI fragment containing the calmodulin-, FMN-, and FAD-binding domains of iNOS was subcloned into pBluescript II SK (Stratagene). The targeting construct, pSPKO-NOS (see Fig. 1), consisted of a 9-kb *Sph* I–*Eco*RI fragment in which the *Apa* I–*Kpn* I fragment containing the calmodulin-binding domain was replaced by the neomycin resistance gene from pMC1Neo polA (27). A copy of the herpes simplex virus thymidine kinase gene was introduced into the construct to allow positive–negative selection (28). Before electroporation, pSPKO-NOS was linearized with *Hpa* I.

ES Cell Transfection and Selection. The 129-derived ES cell line E14TG2a (29) was cultured on irradiated primary embryonic fibroblast-feeder layers in Dulbecco's modified Eagle medium-H, as described (30). Electroporation was essentially as described (31). In 0.8 ml of medium, 3×10^7 ES cells were mixed with 22 μg of the linearized pSPKO-NOS targeting construct and electroporated using the Gene Pulser (Bio-Rad) at 230 V and 500 μF. Cells were then transferred to 10-cm-diameter feeder plates. Medium was replaced the following day with medium containing G418 (200 μg/ml) and ganciclovir (2 μg/ml). Drug-resistant colonies were grown in 16-mm-diameter wells and transferred to 35-mm-diameter wells. Subsequently, half of each culture was lysed for PCR analysis. Recombinants were identified by PCR using the primers a and b as illustrated in Fig. 1. Primer a was 5′-GACAGGTGT-GAGCTACCACATCTGAGTC-3′ and is complementary to a region of the iNOS gene that was not included in the targeting construct, and primer b was 5′-ACTGCTCGACATTGGGT-GGAAACATTCC-3′ and is complementary to the incoming neomycin resistance gene. PCR was performed on 500 ng of genomic DNA for 28 cycles of 1 min at 95°C, 1 min at 60°C,

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; IFN-γ, interferon γ; LPS, lipopolysaccharide; ES, embryonic stem; B6, C57BL/6J; NO_x, nitrite plus nitrate; CALM, calmodulin-binding domain of mouse iNOS cDNA.

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Proc. Natl. Acad. Sci. USA 92 (1995) 10689
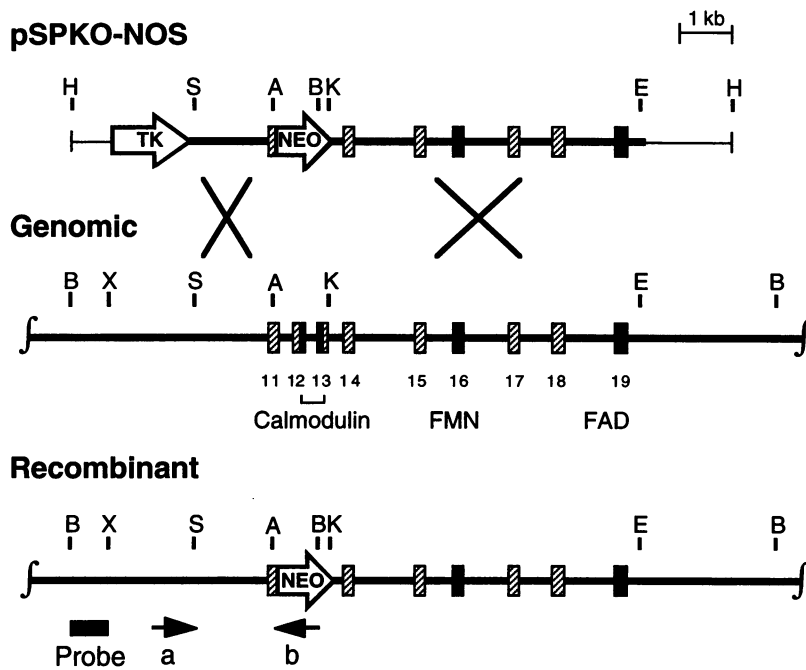
pSPKO-NOS

FIG. 1. Targeted disruption of the mouse iNOS gene. The targeting construct pSPKO-NOS (*Top*), the mouse iNOS genomic region including exons 11–19 (*Middle*), and the recombinant locus (*Bottom*) are illustrated. Exons (represented by boxes) are numbered by analogy to the human iNOS gene (26). Solid boxes indicate cofactor-binding domains, iNOS genomic sequences are indicated by a thick line, and plasmid sequences are indicated by a thin line. Relevant restriction sites are *Apa* I (A), *Bam*HI (B), *Eco*RI (E), *Hpa* I (H), *Kpn* I (K), *Sph* I (S), and *Xmn* I (X). The *Apa* I–*Kpn* I fragment containing the calmodulin-binding domain was replaced by the neomycin resistance gene. Primers specific for an intronic sequence 5' of the targeting vector (arrow a) or the neomycin resistance gene (arrow b) were used for PCR screening of ES cells. The probe used to genotype ES cell and mouse tail DNA by Southern analysis is indicated at the bottom.

and 2 min at 72°C using a DNA thermo cycler (Perkin–Elmer). Southern analysis was used to confirm PCR-identified recombinants.

Generation of Mice Lacking iNOS. To generate chimeric mice, C57BL/6J (B6) blastocysts were injected with the recombinant ES cells and implanted into pseudopregnant females as described (32). Chimeric males were mated with B6 females, and the resulting B6/129 F_1 heterozygotes were mated to generate F_2 mice homozygous for the iNOS disruption. Genotyping of mice was performed by Southern analysis of *Bam*HI-digested tail DNA. Mice were housed under pathogen-free conditions.

The experiments described in this paper used 7- to 10-week-old male and female wild-type (+/+), heterozygous mutant (+/−), and homozygous mutant (−/−) mice from the F_2 generation. Wild-type B6 mice (The Jackson Laboratory) were also used where indicated. In one LPS-induced shock experiment, F_1 (B6/129) mice were used.

Nucleic Acid Analysis. For Southern analysis, 10 μg of genomic DNA from tails was digested with *Bam*HI, electrophoresed on 0.8% agarose gels, and transferred to Zeta-Probe membranes (Bio-Rad). Hybridization was done as recommended by the manufacturer. The probe was a 500-bp *Bam*HI–*Xmn* I genomic fragment (Fig. 1).

For Northern analysis, total RNA was isolated using TRIzol reagent (Life Technologies, Gaithersburg, MD). Samples (10 μg) were electrophoresed on 1% agarose gels containing formaldehyde, transferred to positively charged nylon membranes (Boehringer Mannheim), and hybridized using ExpressHyb solution (Clontech). Probes used in Northern analysis were as follows: cDNA, mouse iNOS cDNA (25); CALM, the calmodulin-binding domain of mouse iNOS cDNA (33); and GAPDH, glyceraldehyde-3-phosphate dehydrogenase cDNA (34).

Macrophage Culture. Macrophages were elicited by treating mice with an i.p. injection of 2 ml of 4% Brewer thioglycollate (Difco). Four days later, cells were harvested by washing out the peritoneal cavity with 20 ml of cold phosphate-buffered saline solution. The cells were washed once with RPMI 1640 medium (Life Technologies)/10% fetal bovine serum and placed in 35-mm-diameter wells (four wells per mouse). After 3 hr at 37°C, nonadherent cells were removed by three washes with complete medium. For induction of iNOS, the adherent

cells were treated with murine IFN-γ at 100 units/ml (Boehringer Mannheim) and LPS at 1 μg/ml (*Escherichia coli* 0111:B4, List Biological Laboratories, Campbell, CA). After 10–12 hr, cells were harvested for the preparation of RNA or cell lysates. Cell lysates were prepared by resuspending cells in 20 mM Tris·HCl, pH 7.5, containing 1 mM dithiothreitol and 10 μM tetrahydrobiopterin, and lysing by three cycles of freeze–thaw.

Immunoblot Analysis. Immunoblot analysis was done on lysates of cultured peritoneal macrophages by conventional procedures using a rabbit polyclonal antibody to murine iNOS (Transduction Laboratories, Lexington, KY) and an alkaline phosphatase-conjugated secondary antibody (Protoblot System, Promega).

NOS Activity and Nitrite/Nitrate Analysis. NOS activity in macrophage lysates was determined by the conversion of ^{14}C-labeled L-arginine to L-citrulline (35) as described (36). Nitrite in macrophage culture supernatants was measured spectrophotometrically as described (36) using the Griess reagent (1% sulfanilamide/0.1% naphthylethylenediamine/2% phosphoric acid). Nitrite plus nitrate (NO_x) was measured in mouse serum samples. To do this, blood was obtained by cardiac puncture, centrifuged after clotting, and deproteinized by filtration through a Millipore Ultrafree-MC (NMWL 30,000) filter unit. Nitrate was enzymatically reduced to nitrite with nitrate reductase (Boehringer Mannheim), and nitrite was then determined spectrophotometrically.

LPS Treatment and Histology of Mice. Mice were injected i.p. with bacterial LPS at 12.5 or 25 mg/kg (*E. coli*, serotype 026:B6, Difco). Tissues were collected from euthanized mice or from mice that died after LPS treatment, fixed in formalin, and stained with hematoxylin/eosin.

RESULTS

Targeted Disruption of Mouse iNOS Gene in ES Cells. The targeting strategy used to inactivate the iNOS gene by homologous recombination is outlined in Fig. 1. After homologous recombination, the calmodulin-binding domain of iNOS is replaced by the neomycin resistance gene, and the reading frame of the modified gene is disrupted. Calmodulin binds tightly to iNOS and is required for electron transfer from NADPH to the iron heme group (37, 38). Removing the

calmodulin-binding domain was therefore expected to result in an inactive iNOS. The targeting construct was introduced into ES cells by electroporation, and clones resistant to both G418 and ganciclovir were isolated and screened by PCR using primers a and b (Fig. 1), which amplify a 1.6-kb product only after homologous recombination. Clones positive by PCR were confirmed by Southern analysis of *Bam*HI-digested genomic DNA. Of 108 doubly-resistant clones screened, 6 contained the desired mutation.

Generation of iNOS −/− Mice. Two targeted ES clones, derived from independent electroporations, were microinjected into 3.5-day-old B6 blastocysts that were then implanted into pseudopregnant mice for development. Male chimeras were bred with B6 females, and offspring heterozygous for the disrupted iNOS gene were identified by Southern analysis of *Bam*HI-digested tail DNA.

To obtain mice homozygous for the disrupted iNOS gene, the F_1 B6/129 mutant heterozygotes were interbred, and their progeny were genotyped by Southern analysis. Fig. 2 shows a representative Southern blot of *Bam*HI-digested genomic DNA; the probe hybridized to a 14-kb fragment from the wild-type allele and to a 5-kb fragment from the mutant allele. Of 266 mice genotyped, 71 (27%) were wild type (+/+), 130 (49%) were heterozygous mutant (+/−), and 65 (24%) were homozygous mutant (−/−), which does not differ significantly from the normal Mendelian distribution ($P = 0.81$ by χ^2 test).

The F_2 mice heterozygous and homozygous for the iNOS disruption were indistinguishable from wild-type F_2 mice in appearance, growth rate, reproduction, and histology. Tissues that were examined included liver, heart, aorta, lung, stomach, intestine, kidney, brain, muscle, pancreas, spleen, salivary gland, thymus, tongue, adrenal gland, and bone.

Analysis of iNOS mRNA in Macrophages from iNOS −/− Mice. The expression of iNOS mRNA in cultured peritoneal macrophages from mice of the F_2 generation (Fig. 3A) was examined. Eleven hours after LPS/IFN-γ treatment, total RNA was prepared and subjected to Northern analysis with two different murine iNOS-specific probes. Both the cDNA and CALM probes hybridized to a single transcript in iNOS +/+ and +/− macrophages. No transcript was detected with the CALM probe in iNOS −/− macrophages. Low levels of two abnormal transcripts were detected with the cDNA probe in iNOS −/− macrophages: one transcript was slightly smaller (≈4 kb), and one was significantly larger (≈5.1 kb) than the 4.4-kb iNOS transcript detected in iNOS +/+ and +/− macrophages. Both abnormal transcripts also hybridized to a neomycin probe (data not shown). These observations confirm that the calmodulin-binding domain was targeted and indicate
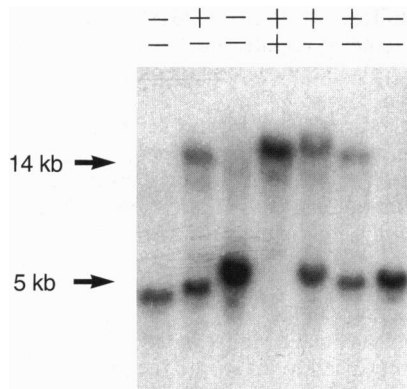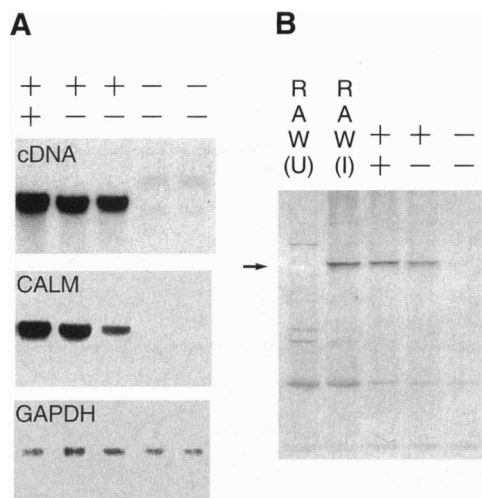

FIG. 3. Expression of iNOS mRNA and protein in F_2 mouse peritoneal macrophages treated with IFN-γ and LPS. (A) Northern analysis. Total RNA (10 μg per lane) from iNOS +/+, +/−, and −/− mouse macrophages was electrophoresed. Hybridization was to the murine iNOS cDNA probe (*Top*), a probe (CALM) specific for the calmodulin-binding sequence of the iNOS cDNA (*Middle*), and a probe specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*Bottom*). (B) Immunoblot analysis. Lysate (10 μg of total protein per lane) was electrophoresed on a SDS/7.5% polyacrylamide gel. iNOS was detected with a rabbit polyclonal antibody to murine iNOS. Lysates were from RAW 264.7 cells either untreated (U) or treated with LPS/IFN-γ (I), and from peritoneal macrophages from iNOS +/+, +/−, and −/− mice treated with LPS/IFN-γ. The arrow shows the band corresponding to the 130-kDa iNOS protein.

that the transcripts detected in iNOS −/− macrophages include sequences from both the iNOS and neomycin resistance genes.

Absence of iNOS Protein in Macrophages from iNOS −/− Mice. Peritoneal macrophages from thioglycollate-treated mice were cultured. Eleven hours after treatment with LPS/IFN-γ, culture supernatants were assayed for nitrite, a stable oxidation product of NO indicative of iNOS activity. The level of nitrite was 70 μM in the culture medium of macrophages from iNOS +/+ mice, 36 μM for +/− macrophages, and <1 μM for −/− macrophages (medium control was <1 μM). All cultures contained approximately equal numbers of cells.

In a more sensitive method for measuring NOS activity, the conversion of ^{14}C-labeled L-arginine to citrulline was determined in lysates of LPS/IFN-γ-treated macrophages. The NOS activity was 950 pmol/min per mg of protein in iNOS +/+ macrophages, 590 in +/− macrophages, and 60 in −/− macrophages. The activity detected in macrophages from iNOS +/+ and +/− mice was inhibited >80% by 50 μM N^G-methyl-L-arginine, a specific inhibitor of NOS, but not by 1 mM EGTA or 50 μM trifluoperazine (a calmodulin antagonist). The low level of activity detected in macrophages from iNOS −/− mice was not inhibited by N^G-methyl-L-arginine, EGTA, or trifluoperazine. Taken together, these results show that the slight activity detected in macrophages from iNOS −/− mice was not due to iNOS, or a constitutive isoform of NOS; it may be due to arginase or some other arginine or citrulline metabolizing enzyme. A similar level of activity was detected in macrophages from iNOS +/+ mice that were not treated with LPS/IFN-γ (data not shown).

To confirm the absence of iNOS protein, immunoblot analysis was done on lysates of LPS/IFN-γ-treated macrophages using a polyclonal antibody to murine iNOS (Fig. 3B). Lysates from iNOS +/+ and +/− macrophages contained the same ≈130-kDa immunoreactive protein that was induced in RAW 264.7 cells, a murine macrophage cell line that expresses iNOS after LPS/IFN-γ treatment (7). In contrast, lysates from

FIG. 2. Southern blot analysis of mouse tail DNA. Genomic DNA (10 μg) was digested with *Bam*HI and hybridized to the indicated probe (Fig. 1). Samples from iNOS +/+, +/−, and −/− mice of the F_2 generation are shown (lane designations). Bands corresponding to wild-type (14 kb) and targeted (5 kb) alleles are indicated.

iNOS $-/-$ macrophages contained no detectable iNOS by immunoblot analysis.

LPS-Induced Shock in iNOS $-/-$ Mice. Generation of NO by iNOS has been thought to play an important role in the pathogenesis of septic shock (22, 39, 40). To evaluate the effects of iNOS deficiency on survival in an LPS-induced model for septic shock, mice were treated i.p. with LPS at either 12.5 or 25 mg/kg, and mortality was monitored over a 96-hr period. The higher dose of LPS was chosen because preliminary tests showed that it led to a mortality rate of >90% in wild-type B6 and in B6/129 F_1 iNOS $+/+$ mice. The lower dose was chosen to allow the assessment of the effects of iNOS genotype when more wild-type survivors would be expected. F_2 mice of the three iNOS genotypes ($+/+$, $+/-$, and $-/-$) were tested at both doses. Wild-type B6 mice were also tested at the high dose.

The results of these experiments are shown in Fig. 4. Each experiment contained equal numbers of mice of each genotype. There were no deaths in saline-treated wild-type B6 mice. At the high dose all LPS-treated wild-type B6 (23 of 23), 90% of F_2 iNOS $+/+$ (18 of 20), 95% of F_2 $+/-$ (19 of 20), and 85% of F_2 $-/-$ mice (17 of 20) died by 96 hr. Neither the $+/-$ nor the $-/-$ mice exhibited a statistically significant survival advantage over the $+/+$ mice ($P = 0.54$ for $+/+$ versus $+/-$ and 0.63 for $+/+$ versus $-/-$ by χ^2 test). At the lower dose, 73% of F_2 iNOS $+/+$ (8 of 11), 64% of $+/-$ (7 of 11), and 91% of $-/-$ mice (10 of 11) died by 96 hr. Again, there was no significant difference in survival among the genotypes ($P = 0.65$ for $+/+$ versus $+/-$ and 0.27 for $+/+$ versus $-/-$ by χ^2 test).

An experiment was also done with B6/129 F_1 iNOS $+/+$ and $+/-$ mice. The F_1 mice provide a completely uniform genetic background for investigating the effect of heterozygosity. These mice were treated with LPS at 25 mg/kg, and as with the F_2 mice, survival of F_1 iNOS $+/-$ mice did not differ significantly from F_1 iNOS $+/+$ mice (11 of 12 $+/+$ and 10 of 12 $+/-$ mice died by 96 hr, $P = 0.76$ by χ^2 test).

Necropsies were done on several F_2 mice of each genotype that died after LPS treatment. No consistent gross pathologic changes were observed. Microscopically, all mice had apoptosis (based on the presence of apoptotic bodies) of lymphocytes in the spleen and thymus. Apoptosis was more extensive in $+/+$ and $+/-$ mice than in $-/-$ mice. Several mice had vacuolar degeneration of centrilobular hepatocytes or degen-
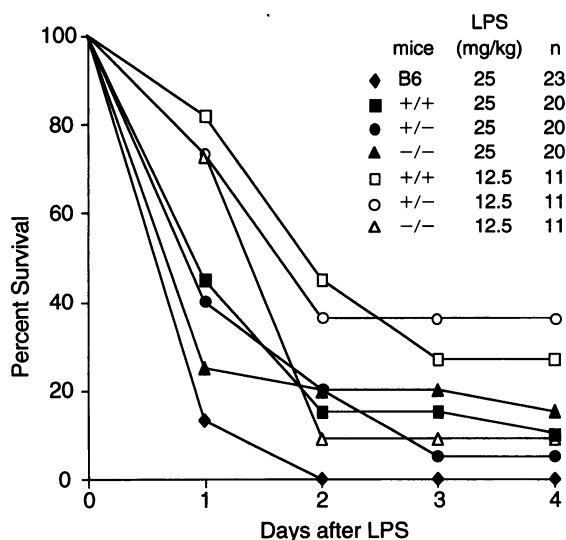

FIG. 4. Survival of LPS-treated mice. B6 mice and iNOS $+/+$, $+/-$, and $-/-$ mice of the F_2 generation were treated i.p. with LPS at either 12.5 mg/kg (open symbols) or 25 mg/kg (closed symbols), and survival was monitored over 4 days.

eration of the adrenal cortex, but neither the incidence nor severity of the changes varied consistently with the genotype.

LPS-Induced Changes in Blood NO$_x$. Wild-type B6 mice and F_2 mice of the three iNOS genotypes were treated with LPS at 25 mg/kg, and four mice from each group were sacrificed 16 hr later. Blood was obtained by cardiac puncture, and nitrite was quantitated in the serum after enzymatic conversion of nitrate to nitrite. The following results were obtained: the serum NO$_x$ level was 16 ± 10 μM for saline-treated wild-type B6 mice, 681 ± 187 μM for LPS-treated wild-type B6 mice, 6 ± 0.5 μM for saline-treated F_2 iNOS $+/+$ mice, 626 ± 175 μM for LPS-treated F_2 $+/+$ mice, 370 ± 119 μM for LPS-treated F_2 $+/-$ mice, and 19 ± 9 μM for LPS-treated F_2 $-/-$ mice. These data show that the F_2 iNOS $-/-$ mice have <4% of the serum NO$_x$ levels of the F_2 iNOS $+/+$ mice after LPS treatment.

DISCUSSION

Although many physiological functions have been ascribed to NO, independent disruptions of the genes for all three NOS isozymes are now known to result in viable, fertile mice. Disruption of the neuronal NOS gene causes no histological abnormalities in the central nervous system, but the stomachs of these mice are enlarged (41). Mice with a disruption of the inducible NOS gene (this paper, and refs. 42 and 43) or the endothelial NOS gene (our unpublished data) are grossly normal.

Enhanced formation of NO by iNOS in the vasculature has been implicated in the pathophysiology of septic shock. Evidence for this has come from observations that serum levels of NO oxidation products (NO$_x$) are elevated in animals and humans undergoing septic shock (39, 44, 45) and from *in vivo* studies with NOS inhibitors (46, 47). However, inhibition of NOS in rodent models for sepsis has produced conflicting results: protective (22, 40), nonprotective (48), and even detrimental effects (49, 50). These studies are complicated by the fact that most of them used a nonselective NOS inhibitor. Therefore concomitant inhibition of either or both of the constitutive isozymes of NOS may have mitigated the effects of iNOS inhibition. We have avoided this complication by generating mice that lack iNOS as a consequence of targeted disruption of the iNOS gene. Our results show that F_2 iNOS $-/-$ mice are not resistant to LPS-induced mortality despite their essential lack of serum NO$_x$ elevations. Likewise, our F_1 iNOS $+/+$ and $+/-$ animals, which are genetically identical except for the iNOS gene disruption, were both sensitive to LPS. This result is despite the fact that the $+/-$ animals have $\approx$50% of the normal level of iNOS activity and $\approx$50% of the serum NO$_x$ levels of wild-type animals after LPS treatment.

Experiments similar to ours have recently been reported by two other groups (42, 43). MacMicking *et al.* (42) observed no differences in survival between iNOS $+/+$ and $-/-$ mice treated with LPS at 5 and 15 mg/kg but a statistically significant (we calculate $P = 0.01$ by χ^2 test) improvement in survival of iNOS $-/-$ over $+/+$ mice treated with LPS at 30 mg/kg. In contrast, our F_2 iNOS $-/-$ mice did not show a statistically significant survival advantage over F_2 iNOS $+/+$ mice after treatment with LPS at either 12.5 or 25 mg/kg. Wei *et al.* (43) found that iNOS deficiency completely prevented death after treatment with LPS at 12.5 mg/kg. In contrast, both MacMicking *et al.* and we observed significant mortality in iNOS $-/-$ mice at a similar LPS dose.

The discrepancies in the data that have just been described are likely to have complex causes. The genetic backgrounds of the mutant mice and the corresponding control mice used in the three studies differed, and, in general, mouse strains differ in their sensitivities to LPS (51). We used F_1 B6/129 ($+/+$ and $-/-$) and F_2 B6/129 ($+/+$, $+/-$ and $-/-$) mice that were genetically equivalent to littermates in our comparisons. Mac-

Micking *et al.* (42) compared B6/129-derived +/+ and −/− animals of an unspecified generation. Wei *et al.* (43) compared MF1/129-derived −/− animals of an unspecified generation with outbred MF1 +/+ animals. Differences in genetic backgrounds consequently appear the most likely explanation for the different results from the three studies. In addition, differences in LPS preparations used in these studies may contribute to discrepancies. However, whatever the final explanation for the differences may prove to be, our experimental results allow the clear statement that iNOS −/− mice, which have virtually no serum NO_x response, are as susceptible to LPS-induced death as their iNOS +/+ littermates that have a normal serum NO_x response. Thus a genetically caused absence of NO produced by iNOS does not prevent mortality in this murine model for septic shock.

1. Knowles, R. G. & Moncada, S. (1994) *Biochem. J.* **298**, 249–258.
2. Nathan, C. & Xie, Q. (1994) *Cell* **78**, 915–918.
3. Bredt, D. S., Hwang, P. M. & Snyder, S. H. (1990) *Nature (London)* **347**, 768–770.
4. Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C. L., Reed, R. R. & Snyder, S. H. (1991) *Nature (London)* **351**, 714–718.
5. Pollock, J. S., Forstermann, U., Mitchell, J. A., Warner, T. D., Schmidt, H. H. H. W., Nakane, M. & Murad, F. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10480–10484.
6. Lamas, S., Marsden, P. A., Li, G. K., Tempst, P. & Michel, T. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 6348–6352.
7. Stuehr, D. J., Cho, H. J., Kwon, N. S., Weise, M. & Nathan, C. F. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 7773–7777.
8. Nussler, A. K., Di Silvio, M., Billiar, T. R., Hoffman, R. A., Geller, D. A., Selby, R., Madariaga, J. & Simmons, R. L. (1992) *J. Exp. Med.* **176**, 261–264.
9. Charles, I. G., Palmer, R. M. J., Hickery, M. S., Bayliss, M. T., Chubb, A. P., Hall, V. S., Moss, D. W. & Moncada, S. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 11419–11423.
10. Scott-Burden, T., Schini, V. B., Elizondo, E., Junquero, D. C. & Vanhoutte, P. M. (1992) *Circ. Res.* **71**, 1088–1100.
11. Snyder, S. H. (1992) *Science* **257**, 4946.
12. Rees, D. D., Palmer, R. M. J. & Moncada, S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 3375–3378.
13. Aisaka, K., Gross, S. S., Griffith, O. W. & Levi, R. (1989) *Biochem. Biophys. Res. Commun.* **160**, 881–886.
14. Hibbs, J. B., Jr., Taintor, R. R., Vavrin, V., Granger, D. L., Drapier, J.-C., Amber, I. J. & Lancaster, J., Jr. (1990) in *Synthesis of Nitric Oxide from a Guanidino Nitrogen of L-Arginine: A Molecular Mechanism That Targets Intracellular Iron*, eds. Moncada, S. & Higgs, E. A. (Elsevier, Amsterdam), pp. 189–223.
15. Nathan, C. F. & Hibbs, J. B., Jr. (1991) *Curr. Opin. Immunol.* **3**, 65–70.
16. Cho, H. J., Xie, Q., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D. & Nathan, C. (1992) *J. Exp. Med.* **176**, 599–604.
17. Yim, C. Y., Bastian, N. R., Smith, J. C., Hibbs, J., Jr. & Samlowski, W. E. (1993) *Cancer Res.* **53**, 5507–5511.
18. Hibbs, J. B., Jr., Taintor, R. R. & Vavrin, Z. (1987) *Science* **235**, 473–476.
19. Stefanovic-Racic, M., Stadler, J. & Evans, C. H. (1993) *Arthritis Rheum.* **36**, 1036–1044.
20. McCartney-Francis, N., Allen, J. B., Mizel, D. E., Albina, J. E., Xie, Q. W., Nathan, C. F. & Wahl, S. M. (1993) *J. Exp. Med.* **178**, 749–754.
21. Miller, M. J., Sadowska-Krowicka, H., Chotinaruemol, S., Kakkis, J. L. & Clark, D. A. (1993) *J. Pharmacol. Exp. Ther.* **264**, 11–26.
22. Kilbourn, R. G., Jubran, A., Gross, S. S., Griffith, O. W., Levi, R., Adams, J. & Lodato, R. F. (1990) *Biochem. Biophys. Res. Commun.* **172**, 1132–1138.
23. Wright, C. E., Rees, D. D. & Moncada, S. (1992) *Cardiovasc. Res.* **26**, 48–57.
24. Parillo, J. E. (1993) *N. Engl. J. Med.* **328**, 1471–1477.
25. Lyons, C. R., Orloff, G. J. & Cunningham, J. M. (1992) *J. Biol. Chem.* **267**, 6370–6374.
26. Chartrain, N. A., Geller, D. A., Koty, P. P., Sitrin, N. F., Nussler, A. K., Hoffman, E. P., Billiar, T. R., Hitchinson, N. I. & Mudgett, J. S. (1994) *J. Biol. Chem.* **269**, 6765–6772.
27. Thomas, K. R. & Capecchi, M. R. (1987) *Cell* **51**, 503–512.
28. Mansour, S. L., Thomas, K. R. & Capecchi, M. R. (1988) *Nature (London)* **336**, 348–352.
29. Hooper, M., Hardy, K., Handyside, A., Hunter, S. & Monk, M. (1987) *Nature (London)* **326**, 292–295.
30. Koller, B. H., Kim, H.-S., Latour, A. M., Brigman, K., Boucher, R. C., Jr., Scambler, P., Wainwright, B. & Smithies, O. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10730–10734.
31. Piedrahita, J. A., Zhang, S. H., Hagaman, J. R., Oliver, P. M. & Maeda, N. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 4471–4475.
32. Koller, B. H. & Smithies, O. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 8932–8935.
33. Xie, Q., Cho, H. J., Calaycay, J., Mumford, R. A., Swiderek, K. M., Lee, T. D., Ding, A., Troso, T. & Nathan, C. (1992) *Science* **256**, 225–228.
34. Dugaiczyk, A., Haron, J. A., Stone, E. M., Dennison, E., Rothblum, K. N. & Schwartz, R. J. (1983) *Biochemistry* **22**, 1605–1613.
35. Bredt, D. S. & Snyder, S. H. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 9030–9033.
36. Sherman, P. A., Laubach, V. E., Reep, B. R. & Wood, E. R. (1993) *Biochemistry* **32**, 11600–11605.
37. Abu-Soud, H. M. & Stuehr, D. J. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 10769–10772.
38. Nathan, C. & Xie, Q. (1994) *J. Biol. Chem.* **269**, 13725–13728.
39. Goode, H. F., Howdle, P. D., Walker, B. E. & Webster, N. R. (1995) *Clin. Sci.* **88**, 131–133.
40. Szabo, C., Southan, G. J. & Thiemermann, C. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 12472–12476.
41. Huang, P. L., Dawson, T. M., Bredt, D. S., Snyder, S. H. & Fishman, M. C. (1993) *Cell* **75**, 1273–1286.
42. MacMicking, J. D., Nathan, C., Hom, G., Chartrain, N., Fletcher, D. S., Trumbauer, M., Stevens, K., Xie, Q.-w., Sokol, K., Hutchinson, N., Chen, H. & Mudgett, J. S. (1995) *Cell* **81**, 641–650.
43. Wei, X.-q., Charles, I. G., Smith, A., Ure, J., Feng, G.-j., Huang, F.-p., Xu, D., Muller, W., Moncada, S. & Liew, F. Y. (1995) *Nature (London)* **375**, 408–411.
44. Barthlen, W., Stadler, J., Lehn, N. L., Miethke, T., Bartles, H. & Siewert, J. R. (1994) *Shock* **2**, 398–401.
45. Cunha, F. Q., Assreuy, J., Moss, D. W., Rees, D., Leal, L. M. C., Moncada, S. & Carrier, M. (1994) *Immunology* **81**, 211–215.
46. Petros, A., Bennett, D. & Vallance, P. (1991) *Lancet* **338**, 1557–1558.
47. Tracey, W. R., Tse, J. & Carter, G. (1995) *J. Pharmacol. Exp. Ther.* **272**, 1011–1015.
48. Evans, T., Carpenter, A., Silva, A. & Cohen, J. (1994) *J. Infect. Dis.* **169**, 343–349.
49. Billiar, T. R., Curran, R. D., Harbrecht, B. G., Stuehr, D. J., Demetris, A. J. & Simmons, R. L. (1990) *J. Leuk. Biol.* **48**, 565–569.
50. Minnard, E. A., Shou, J., Naama, H., Cech, A., Gallagher, H. & Daly, J. M. (1994) *Arch. Surg.* **129**, 142–148.
51. Heppner, G. & Weiss, D. W. (1965) *J. Bacteriol.* **90**, 696–703.